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Award Number: DAMD17-01-1-0477

TITLE: Inhibition of Estrogen Receptor Coactivator Expression by Antisense Oligodeoxynucleotides and Effect on Breast Cancer Cell Proliferation and Gene Expression

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REPORT DATE: May 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2002	Annual (23 Apr 01 - 22 Apr 02)

4. TITLE AND SUBTITLE
Inhibition of Estrogen Receptor Coactivator Expression by Antisense Oligodeoxynucleotides and Effect on Breast Cancer Cell Proliferation and Gene Expression

5. FUNDING NUNUMBER
DAMD17-01-1-0477

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Coactivators are nuclear proteins that interact with steroid receptors, such as estrogen receptor-α (ERα), and are required for the ability of receptors to stimulate the expression of target genes. Antiestrogen ligand are commonly utilized in the treatment of breast cancer to negatively regulate the activity of steroid receptors. However, tumors often can develop resistance to antiestrogen therapy. Therefore, as an alternative approach to inhibiting ERα function in breast cancer cells, we have developed antisense oligonucleotides against three of the major ERα coactivator proteins. These oligonucleotides decrease the expression of coactivator mRNA and protein, and in so doing, decrease the ability of ERα to stimulate gene expression. These oligonucleotides also decrease the proliferation of MCF-7 breast cancer cells in response to estrogen treatment. Taken together, antisense oligonucleotide technology has the potential to regulate ERα action at a level that circumvents ligand control, and therefore represents a novel mechanism by which to inhibit breast cancer gene expression and proliferation, and potentially to regulate the growth of breast cancer.

14. SUBJECT TERMS
breast cancer, endocrinology, estrogen receptor, coactivators

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Although estrogens are important for normal breast development, they also have been linked to breast cancer, at least in part through their ability to stimulate cell proliferation. Inhibition of the ability of ER to mediate estrogen action therefore has been a major goal in the treatment, and more recently prevention of hormone-dependent breast cancer. Estrogen effects are mediated by two, high affinity intracellular receptor proteins, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), that are members of a superfamily of transcription factors. Once activated by ligands, these receptors interact with coactivators that positively enhance ER transcriptional activity by acting as bridging factors to the general transcriptional machinery as well as modifying local chromatin structure. Amongst the best characterized coactivators that interact with ER are members of the steroid receptor coactivator family of coactivators (SRC-1, SRC-2/TIF2 and SRC-3/RAC3/AIB1). Recently it was determined that AIB1 (SRC-3) was amplified in 7% of human breast tumors, and that overexpression of AIB1 correlated with increased tumor size, suggesting a important role for this coactivator in regulating breast cell growth and possibly tumorigenesis. Furthermore, targeted deletion of the gene for SRC-1 in mice results in generalized resistance to steroid hormones and underdeveloped mammary glands characterized by reduced ductal branching and lobuloaveolar development thus reinforcing the concept that coactivators are required for efficient ER-mediated biological responses *in vivo*. Tamoxifen therapy is used in the prevention and more commonly, the treatment of breast cancer. Its biological effects are related to its ability to inhibit ER action. However, in advanced disease, tamoxifen therapy fails, and may even promote tumor growth. It is interesting to note that the experimental evidence to date indicates that when tamoxifen stimulates ER action, it does so in conjunction with ER coactivator proteins. Thus coactivators are a common mediator of ER action regardless of whether estrogen or tamoxifen is present, and they therefore represent a more universal target for inhibiting ER action than alternative antiestrogen (ligand-based) therapies.

Body

Antisense Oligodeoxynucleotides. Most cellular studies on the functions of coactivators and corepressors have relied upon their overexpression followed by subsequent characterization of the event of interest. While this approach has provided solid information on the potential role of these molecules in ERα action, the results may lack specificity due to overexpression. This obstacle could be overcome in cell lines lacking expression of the target of interest (*e.g.* mouse embryo fibroblasts from knock-out mice), but these cells limit the scope of biological processes that can be examined and may be influenced by compensatory overexpression of other coactivators as seen in the SRC-1 knock-out mouse[1]. Therefore, in collaboration with ISIS Pharmaceuticals, we have developed antisense oligonucleotides that significantly and specifically inhibit the expression of a series of coregulators including SRC-1, TIF2 and RAC3. This is possible because oligonucleotides of up to 20 bases are able to discriminate between two gene products that differ by a single base[2]. These oligonucleotides utilize a unique chemistry that yields a stable and effective inhibitor of target gene mRNA and protein expression. These oligonucleotides are also very specific for the target of interest. For example, in cells treated with a TIF2 antisense oligonucleotide, only TIF2 and not SRC-1 mRNA, is down regulated (**Fig. 1A**). Expression is also reduced at the protein level as demonstrated by the ability of SRC-1, but

not TIF2 antisense oligonucleotides to reduce SRC-1 protein expression (**Fig. 1, C&D**). Importantly, we have shown that suppression of mRNA levels can be maintained for up to 72 hours (**Fig. 2**), and this agrees with other published reports using oligonucleotides with this unique composition[3]. We also have tested antisense oligonucleotides against RAC3, which effectively suppress its mRNA expression (**Fig. 3**). Using FITC-labeled oligonucleotides, we have demonstrated that oligonucleotides are taken up by virtually 100% of the cells (**Fig. 4**). We have also shown that oligonucleotides against SRC-1, TIF2 and SRA inhibit ERα transcriptional activity measured on a 3xERE-TATA-Luc reporter as well as coactivator expression in a dose-dependent manner in HeLa cells (**Fig. 5**).

Using antisense oligonucleotide technology, we proposed to examine the role of these specific coactivators in regulating ERα activity. We chose the MCF-7 cell as a starting point for these studies because these cells are ERα-positive, enabling us to perform experiments without having to transfect ERα expression plasmid. This also permitted an examination of the role of specific coactivators on well characterized ER-mediated events, such as induction of gene regulation or stimulation of cell growth. Our results demonstrate the impact of decreasing coactivator expression on estrogen induction of pS2 mRNA and find that antisense oligonucleotides against SRC-1 and TIF2, but not SRA inhibit pS2 gene expression (**Fig. 6A**). Parallel analyses of these same RNA samples for coactivator mRNA levels verify that the antisense oligonucleotides decrease their expression as expected (**Fig. 6B**). This contrasts with the ability of antisense oligonucleotides against each of these coactivators to inhibit expression of a transiently transfected ER target gene in HeLa cells (see above). Taken together, these results suggest the interesting possibility that the importance of specific coactivators to ERα activity may vary depending on the specific target gene (ERE-TATA-CAT *versus* pS2), whether the target is endogenous or transiently expressed, or perhaps by cell type (HeLa *versus* MCF-7 cells). With the development of these novel oligonucleotides, we are well positioned with the research tools to examine the contribution of coactivators to ligand-specific, gene-specific and cell-specific regulation of ERα function.

Effects of Coactivator Antisense Oligonucleotides on MCF-7 Cell Growth. MCF-7 cells express ERα and their growth is estrogen-dependent[4]. Therefore, in order to determine if any of the coactivators examined in our studies contributed to estrogen-mediated growth, MCF-7 cells were transfected with the indicated quantity of *as*ODNs or their corresponding *rs*ODNs, and twenty-four hours thereafter cell proliferation was assessed by [^{3}H]thymidine incorporation. It is important to note that these studies are possible because virtually all the cells uptake ODN. As shown in **Fig. 7A**, *as*ODNs to SRC-1 or TIF2 decreased cell proliferation in comparison to cells transfected with appropriate levels of *rs*ODN. Interestingly, SRA *as*ODN did not inhibit [^{3}H]thymidine incorporation in these cells, but instead had a modest stimulatory effect on DNA synthesis. These results were further substantiated in cells grown in stripped serum in the absence or presence of 1 nM E2 to ensure that the *as*ODN inhibited estrogen-induced cell proliferation (**Fig. 7B**). Estradiol stimulated [^{3}H]thymidine incorporation in SRA, SRC-1 or TIF2 *rs*ODN-treated cells by 4-5 fold. These increases in DNA synthesis were attenuated in cells treated with *as*ODNs to either SRC-1 or TIF2, but not to SRA; a result similar to that obtained for Fig. 7A.

Cell Models of Selective 4HT's Agonist/Antagonist Activity. We have obtained MCF7/LCC1 and MCF7/LCC2 cells from Dr. Robert Clarke at the Lombardi Cancer Research Center

(Georgetown University). As will be explained below, these cells are derived from MCF-7 (human breast) cells, which require estrogen for their growth and are growth inhibited by 4HT. The derivative cell lines, which will be referred to as LCC1 and LCC2, have significant alterations in their growth responses to E2 and 4HT[5,6]. However, since these cells are from a common lineage, the relative differences between them should be biologically, and will provide information about alternations in the expression and activity of coactivators and their functions that occur as breast cancer cells progress to a hormone-independent, but hormone-responsive phenotype.

The LCC1 cells were derived from MCF-7 cells that had been passaged twice in ovariectomized athymic nude mice[5]. These cells do not require E2 for growth in culture and are therefore considered to be hormone-independent[5,6]. However, like the MCF-7 parental line, they are growth inhibited by 4HT as well as the pure antiestrogens, ICI 164,384 and ICI 182,780[5,7]. The LCC1 cell line also exhibits a more malignant phenotype that has been attributed to altered gene regulation rather than gene amplification[5]. Stepwise selection of LCC1 cells in increasing concentrations of 4HT produced the LCC2 cell line which grows independently of estrogen and is resistant to growth inhibition by 4HT *in vitro* and *in vivo*[6]. Importantly, these cells also retain sensitivity to ICI 182,780 and ICI 164,384[6,8] indicating that they still possess functional ER responses, and that this resistance is specific to 4HT (**Fig. 8**). None of these cells express detectable aromatase activity[9], but all express equivalent levels of ER[5]. In each cell line, the E2-induction of pS2 gene expression by estrogen, considered to be a primary estrogenic response [10] and cathepsin D is retained[5,8,11]. We are now prepared to examine the impact of decreasing coactivator expression in these cell lines.

Key Research Accomplishments

1. Development and verification of antisense oligonucleotides against the coactivators, SRC-1, TIF2 and RAC3.
2. Oligonucleotides against SRC-1, TIF2 and SRA reduce the transcriptional activity of ERα in HeLa cells.
3. Oligonucleotides against SRC-1 and TIF2 reduce the expression of the estrogen target gene, pS2 in MCF-7 cells.
4. Oligonucleotides against SRC-1 and TIF2 reduce the estrogen-dependent growth of MCF-7 cells.
5. Inhibition of cell growth and induction of pS2 expression is specific to certain coactivators, since reductions in the expression of the SRA coactivator, did not inhibit MCF-7 cells growth or estrogen induction of pS2 mRNA expression.

Reportable Outcomes

1. Funding applied for and received, based in part on this work – "Coactivator antisense oligonucleotides as therapeutic targets for breast cancer." Funded by the State of Texas Advanced Technology Program – Biomedicine (01/01/02 – 12/31/03).

Conclusions

Our work to date indicates that antisense oligonucleotides against SRC-1, TIF2 and RAC3 (SRC family coactivators) decreases the expression of the mRNAs and proteins for these targets. This provides reagents that have enabled us to explore the role of these coactivators in regulation of estrogen-dependent cell growth and proliferation. This clearly established these coactivators as important *in vivo* determinants of these biological processes, and advances the principle that they may be targeted for affecting breast cancer cell growth. We continue to explore this avenue of investigation.

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Appendixes

Please see figures on pages 8 to 15.

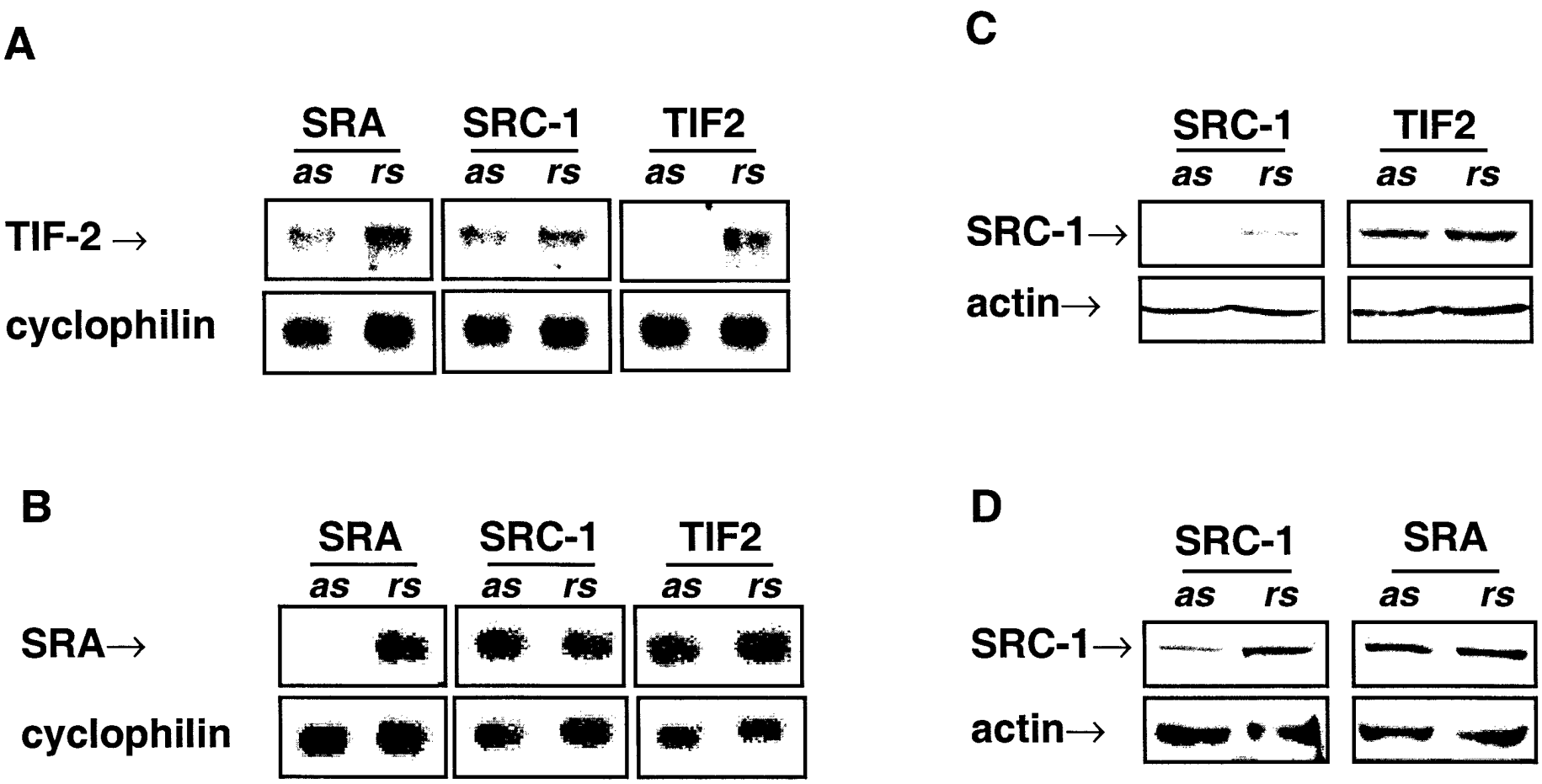

Figure 1: SRA, SRC-1 and TIF2 *as*ODNs are specific for their mRNA/protein targets. **A)** Northern blot analysis of total RNA extracted from cells treated for 4 hours with 100 pmol of *as*ODN or *rs*ODN for SRA, SRC-1 or TIF2 and harvested 24 hours later. TIF2 and cyclophilin mRNAs are indicated. **B)** Cells treated with 200 pmol of the same asODNs and rsODNs were similarly subjected to Northern analysis for SRA expression. **C)** Western analysis of proteins extracted from cells treated for 4 hours with 100 pmol of *as*ODN or *rs*ODN for SRC-1 or TIF2 and harvested 24 h later. SRC-1 and actin proteins are indicated. **D)** Western analysis of proteins extracted from cells treated for 4 hours with 50 pmol of SRC-1 *as*ODN, SRC-1 *rs*ODN, SRA *as*ODN or SRA *rs*ODN and harvested 24 h later. SRC-1 and actin proteins are indicated.

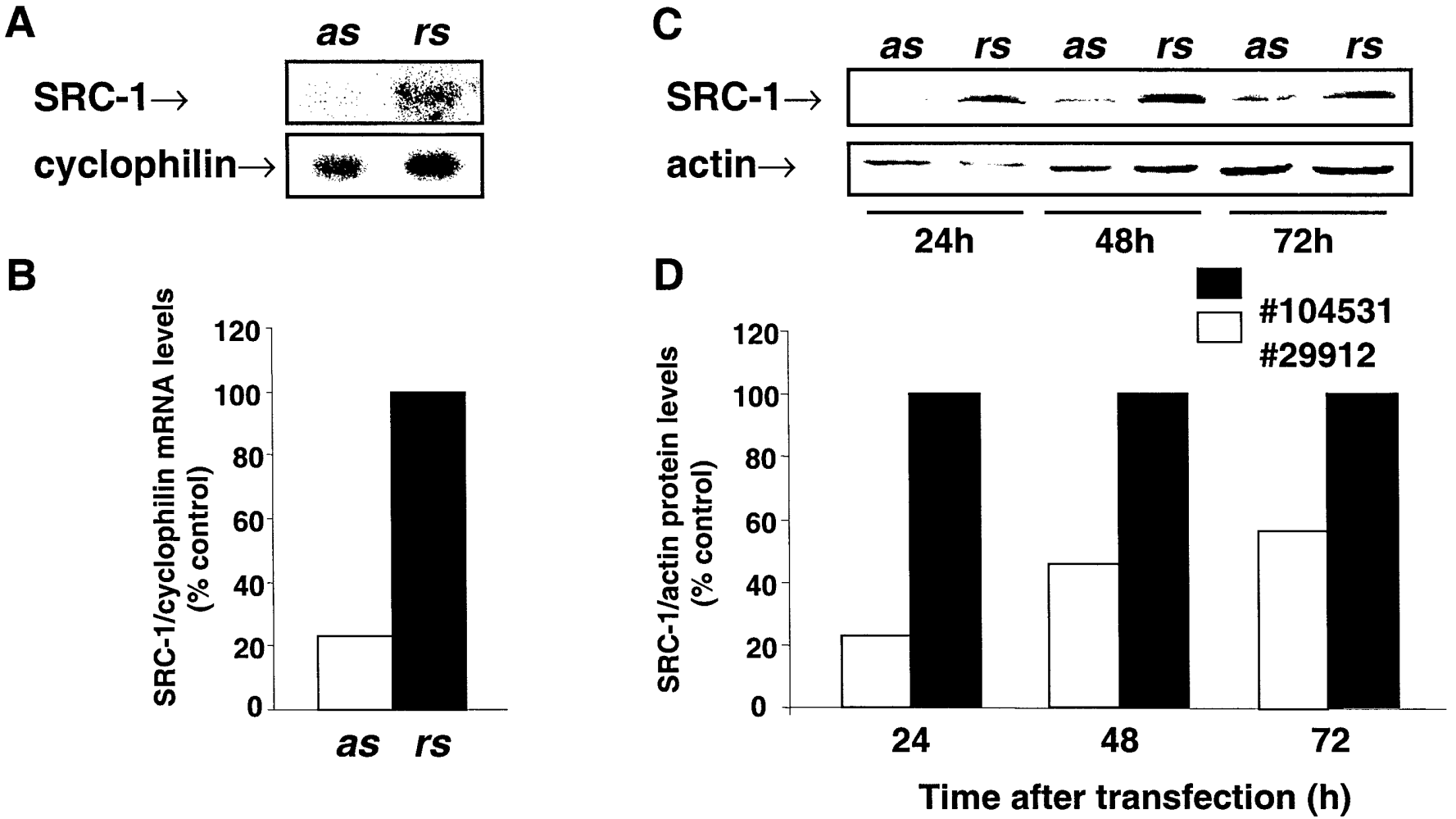

Figure 2: Effect of SRC-1 *as*ODN on SRC-1 mRNA and protein expression. **A)** Northern blot analysis of total RNA extracted from cells treated for four hours with 200 pmol of SRC-1 *as*ODN (#29912) or *rs*ODN (#104531) and harvested 24 hours later. SRC-1 and cyclophilin mRNAs are indicated. **B)** Quantification of the Northern blot by scanning laser densitometry. SRC-1 mRNA levels in the presence of the *as*ODN (open bar) are corrected to cyclophilin mRNA levels and expressed as percentage of the SRC-1 mRNA levels measured in the presence of an equivalent quantity of the corresponding *rs*ODN (solid bar). **C)** Western blot analysis of proteins extracted from cells treated with 200 pmol SRC-1 *as*ODN or *rs*ODN for four hours and harvested 24, 48 or 72h thereafter. SRC-1 and actin proteins are indicated. **D)** Quantification of the Western blot by scanning laser densitometry. SRC-1 protein levels in the presence of the *as*ODN (open bar) are corrected by actin protein levels and expressed as percentage of the protein levels measured in the presence of equivalent amounts of the corresponding *rs*ODN (solid bar).

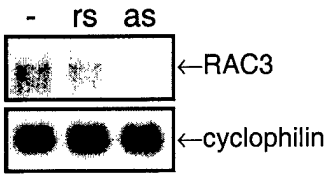

Figure 3: RAC3 antisense oligonucleotides inhibit RAC3 mRNA expression. HeLa cells were treated with 100 pmol of random or antisense (rs or as, respectively) oligonucleotides or left untreated ('-'). RNA was extracted 24 h thereafter and subjected to Northern blot analyses for RAC3 (*top*) and cyclophilin (*bottom*) mRNA levels.

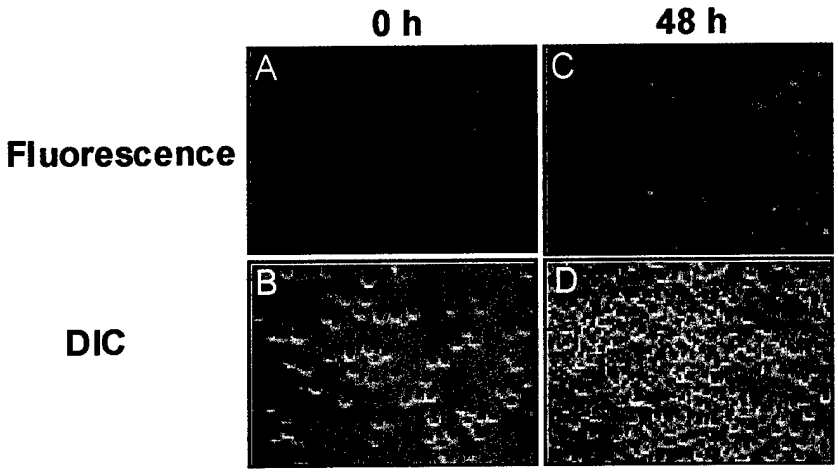

Figure 4. Uptake of a FITC-conjugated ODN by HeLa cells. Cells were transfected with a fluorescein-conjugated ODN using Lipofectamine. The efficiency of transfection was evaluated by observing the cell fluorescence pattern immediately after removing the Lipofectamine/ODN mixture (**A**) and 48 hours thereafter (**C**) and by comparing the number of fluorescent cells to the total number of cells observed by DIC-microscopy (**B and D,** respectively).

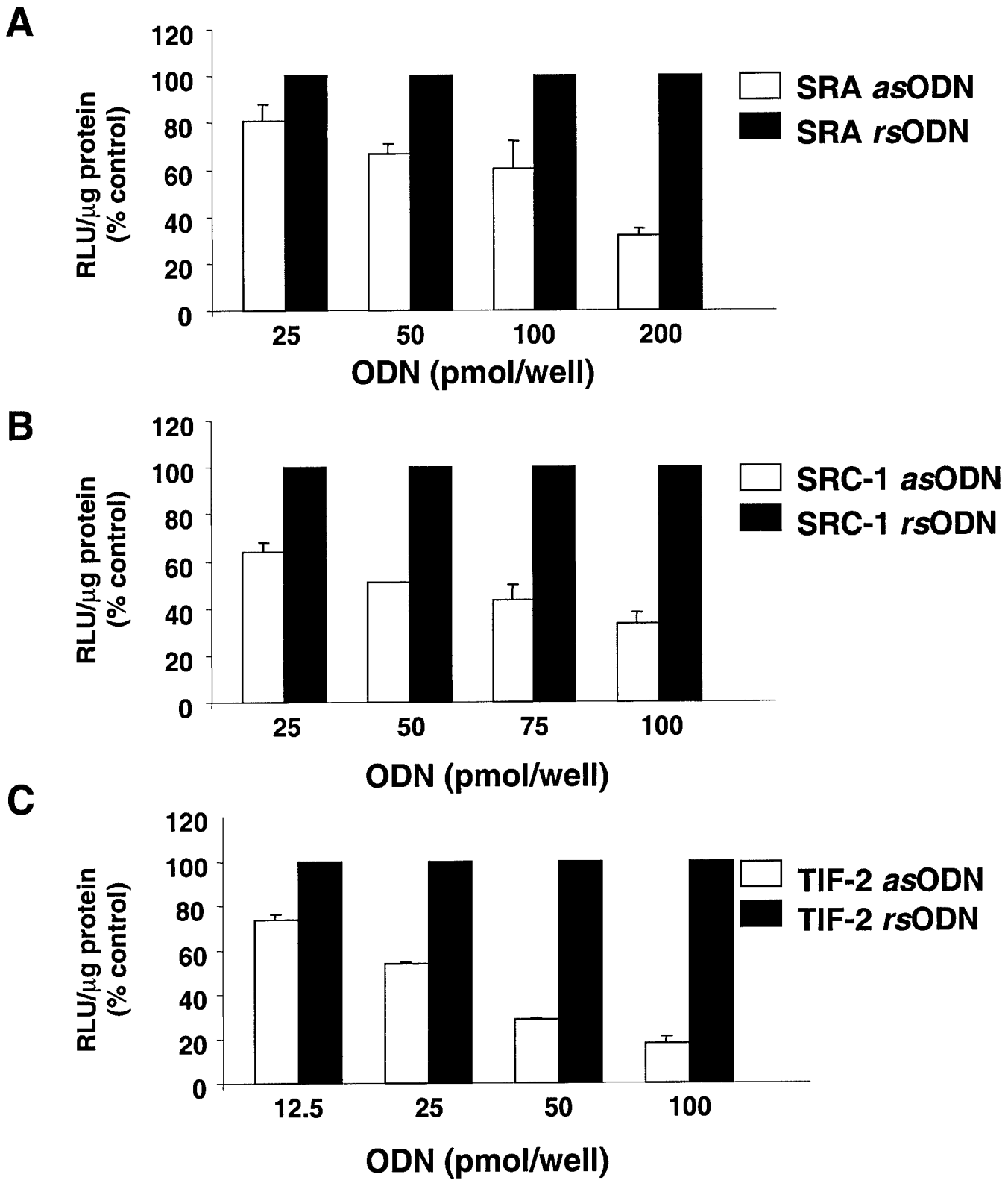

Figure 5: ERα-transcriptional activity is impaired in a dose-dependent manner by the presence of SRA, SRC-1 and TIF-2 *as*ODNs. **A)** Cells were transfected for two hours with 25, 50, 100 or 200 pmol of SRA *as*ODN (open bar) or with equivalent quantities of the corresponding *rs*ODN (solid bar) along with $pCMV_5hER\alpha$ and a 3xERE-TATA-Luciferase target gene, and treated with 1 nM estradiol. Luciferase activity represents the mean of duplicate samples obtained from cells treated with *as*ODN expressed as a percentage of the RLU from cells treated with the *rs*ODN. Each plot represents one of at least three independently repeated experiments. Values from cells treated with *as*ODN or *rs*ODN for SRC-1 and with the *as*ODN or the *rs*ODN for TIF2 are shown in **B** and **C**, respectively.

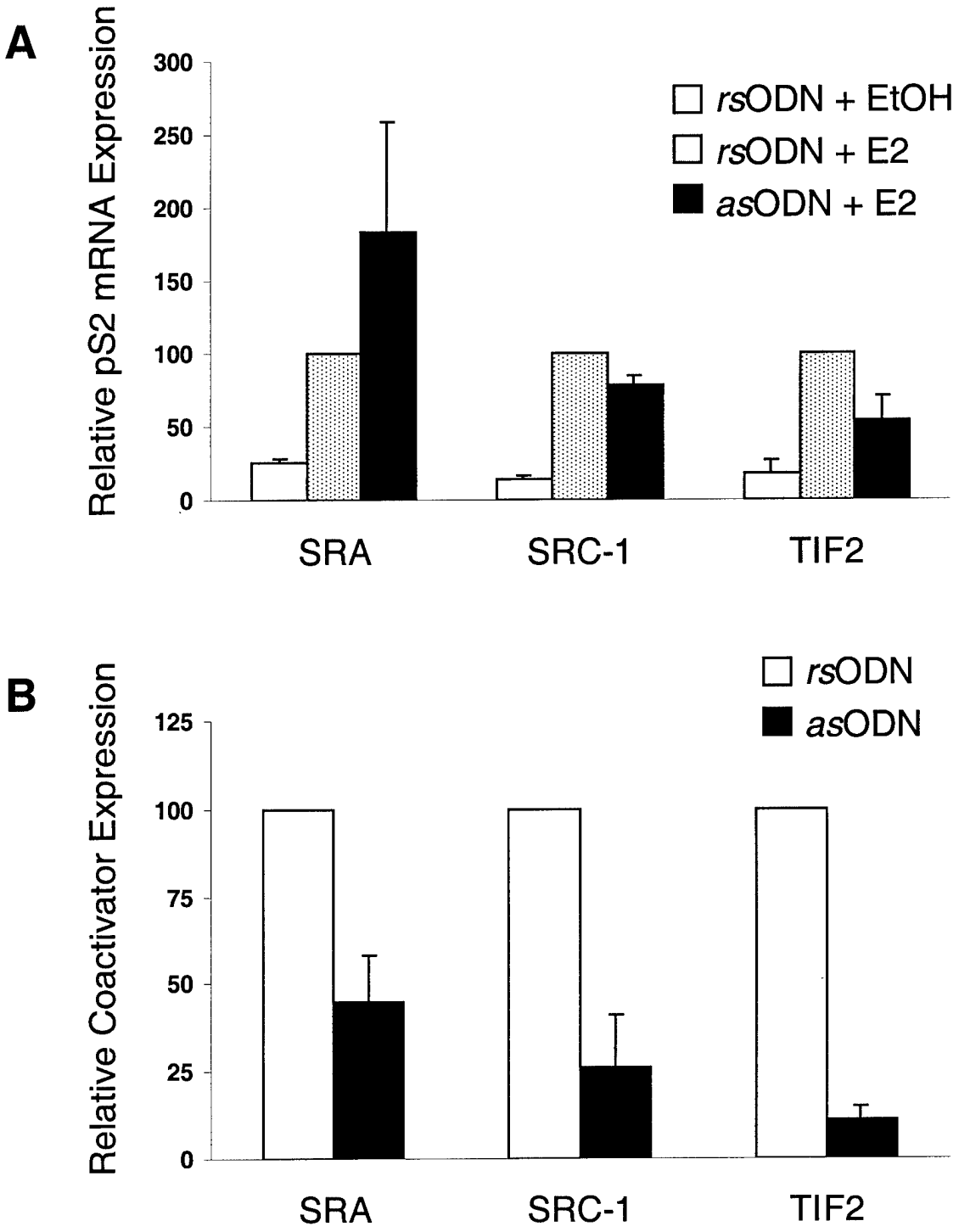

Figure 6: Inhibition of estrogen-induced pS2 mRNA expression by asODNs is coactivator-specific. **A)** MCF-7 cells grown in media containing 5% sFBS were transfected with 200 pmol of *rs*ODN or *as*ODN for the indicated coactivators and treated 24 h thereafter with 0.1% ethanol vehicle (EtOH) or 1 nM E2 for 16 h prior to harvesting cells for RNA isolation and quantitative pS2 and 18S RNA measurements by real-time RT-PCR. Values are presented relative to the pS2 mRNA levels normalized to 18S RNA values determined for estrogen and *rs*ODN treated samples (=100), and are given as the mean ± SEM for 3-5 independent experiments. **B)** Effect of *as*ODN on coactivator mRNA expression. MCF-7 cells were transfected with 200 pmol of *rs*ODN or *as*ODN for the indicated coactivator, and harvested 40 h later for RNA isolation and coactivator and 18S RNA measurements by real-time RT-PCR. Values are presented relative to the coactivator levels normalized for 18S levels determined for *rs*ODN treated samples (=100), and are given as the mean ± SEM for 3-6 independent experiments.

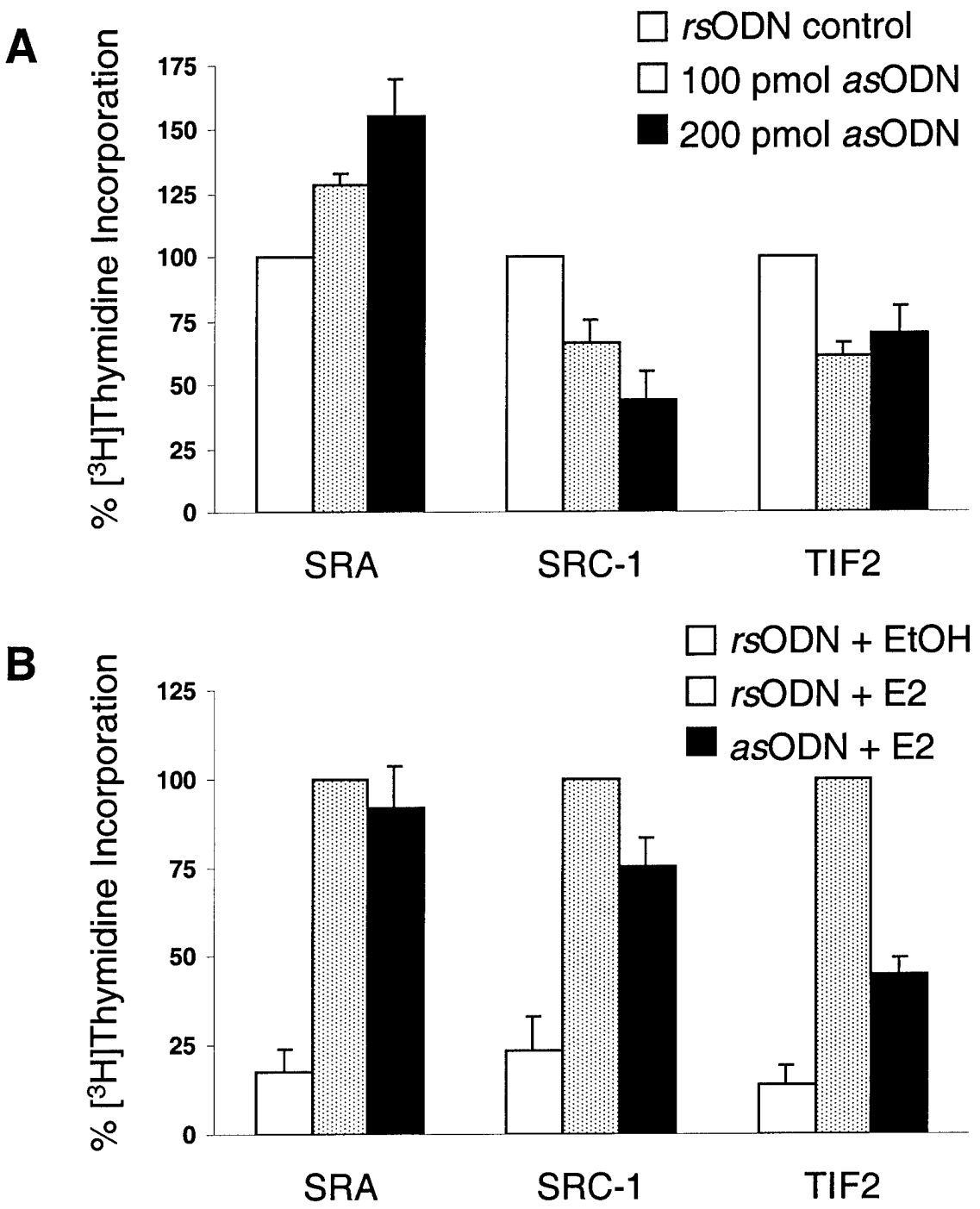

Figure 7: Inhibition of MCF-7 DNA synthesis by *as*ODNs is coactivator-specific. (**A**) Cells grown in media containing 10% FBS were treated with the indicated amounts of *as*ODNs and 24 hours after transfection, cell proliferation was assessed by [^{3}H]thymidine incorporation. Values are calculated as the percentage of incorporated counts in *as*ODN-treated cultures in comparison to the counts obtained in cultures transfected with the corresponding amount of *rs*ODN, and are given as the mean ± SEM for 3-4 independent experiments. (**B**) Cells grown in media containing 5% sFBS were transfected with 200 pmol of *rs*ODN or *as*ODN for the indicated coactivators and treated 24 h thereafter with ethanol vehicle (EtOH) or 1 nM E2 for 16 h to induce DNA synthesis. Values are calculated relative to the percentage of incorporated counts in *rs*ODN and E2 treated cultures (100%) for each coactivator, and are given as the mean ± SEM for 3-4 independent experiments.

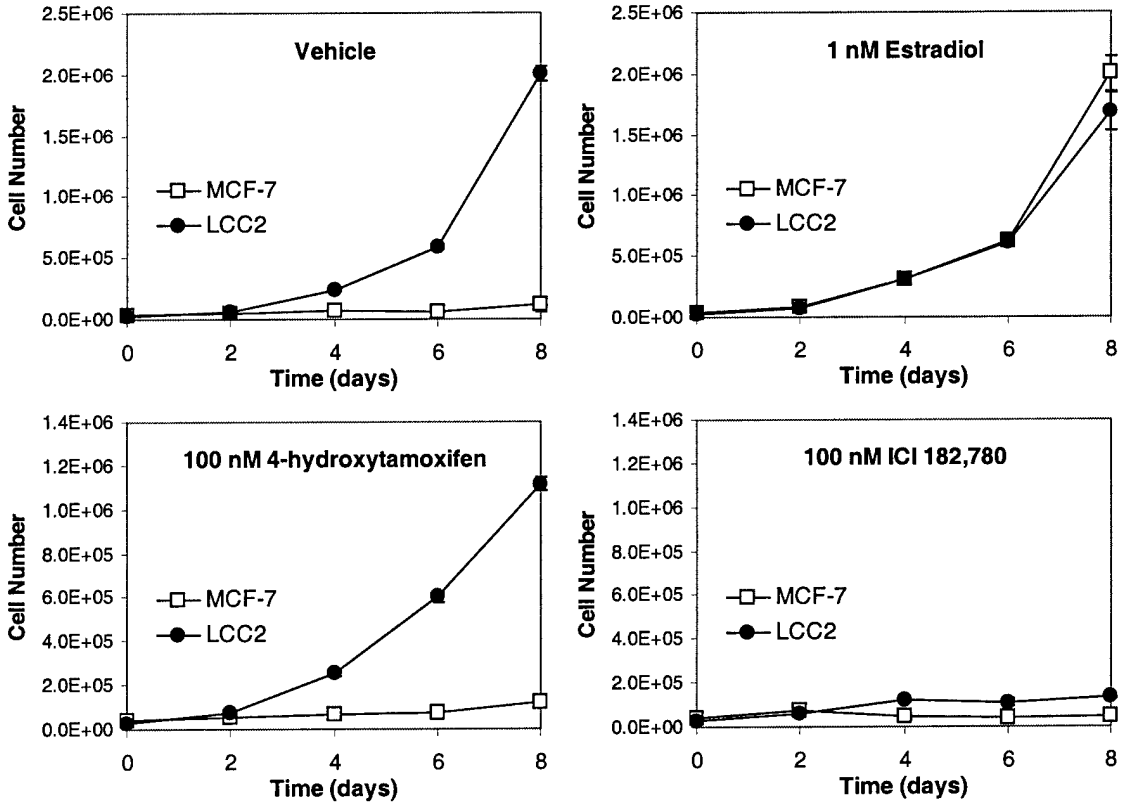

Figure 8: Effect of hormone treatment on growth of MCF-7 and LCC2 cells grown in phenol-red free DMEM & 10% sFBS.